Exhibit M

ARTIFICIAL DENTAL PLAQUE BIOFILM MODEL SYSTEMS

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Abstract—Difficulties with *in vivo* studies of natural plaque and its complex, heterogeneous structure have led to development of laboratory biofilm plaque model systems. Technologies for their culture are outlined, and the rationale, strengths, and relative uses of two complementary approaches to microbial models with a focus on plaque biodiversity are analyzed. Construction of synthetic consortia biofilms of major plaque species has established a variety of bacterial interactions important in plaque development. In particular, the 'Marsh' nine-species biofilm consortia systems are powerful *quasi* steady-state models which can be closely specified, modified, and analyzed. In the second approach, microcosm plaque biofilms are evolved *in vitro* from the natural oral microflora to the laboratory model most closely related to plaque *in vivo*. Functionally reproducible microcosm plaques are attainable with a biodiverse microbiota, heterogeneous structure, and pH behavior consistent with those of natural plaque. The resting pH can be controlled by urea supply. Their growth patterns, pH gradient formation, control of urease levels by environmental effectors, and plaque mineralization have been investigated. Microcosm biofilms may be the only useful *in vitro* systems where the identity of the microbes and processes involved is uncertain. Together, these two approaches begin to capture the complexity of plaque biofilm development, ecology, behavior, and pathology. They facilitate hypothesis testing across almost the whole range of plaque biology and the investigation of antiplaque procedures yielding accurate predictions of plaque behavior *in vivo*.

Key words: plaque, microcosm, biofilm, oral bacteria, consortia, culture technology.

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LABORATORY MODELING AND THE NATURE OF DENTAL PLAQUE

Why model dental plaque?

In vivo experimental studies of natural dental plaque in both humans and animals are severely restricted. Complicated ethical issues, including induction of gingivitis by simple plaque accumulation, and problems with access and sampling present considerable difficulties. Plaque is a microbial biofilm defined only by its location on teeth: Smooth-surface, fissure, approximal, and subgingival plaques are recognized as distinct (Marsh and Martin, 1992). Plaque has a biodiverse, heterogenous, organized structure changing over time (Listgarten, 1994), yet displaying considerable homeostasis, and is sited in an almost uncontrollable, fluctuating locus-specific oral environment (Table 1; Bowden and Li, 1997; Caldwell *et al.*, 1997; Marsh and Bradshaw, 1997; Wimpenny, 1997). Dental disease is correspondingly also site-specific (Dawes and Macpherson, 1993). As a result of these complications, *in vivo* experimental protocols are limited, variability high, and interpretation of results difficult.

Model systems which are more controllable than natural dental plaque have been developed and then used to explain and predict its behavior (Tatevossian, 1988; Gilbert and Allison, 1993; Wimpenny *et al.*, 1993; Bowden, 1995; Marsh, 1995a; Wimpenny, 1997). There are considerable difficulties inherent in the modeling of such a heterogeneous and variable biofilm. To explain plaque ecology, pathology, and behavior, the model must be realistic, reflecting the very behavior which is under investigation, predicting plaque behavior *in vivo* in response to perturbation and interventions to prevent dental disease. A range of technologies and microbial systems has been utilized, all with different uses, strengths, and limitations. All are compromises between the reality of the *in vivo* ecosystem and the simplification and controllability necessary to gain meaningful, useful results.

The quest for biodiversity: defined species consortia and plaque microcosms

A key feature of plaque is its high genetic heterogeneity or biodiversity, with probably 30 to 300 species in 500-plus taxa, and an unknown number of genetically distinct subspecies strains (clonotypes) of each (Moore and Moore, 1994). These microflora vary widely between intra-oral sites and people (Marsh 1989, 1991a) and over time (Socransky *et al.*, 1977). Such biodiversity may insure against changes in the environment and enhance ecosystem resistance to adversity (Naeem *et al.*, 1994; Tilman and Downing, 1994). An understanding of the role of this great biodiversity is necessary if we are to predict and control the appearance of species and clonotypes which have a major impact on plaque

TABLE 1

ASPECTS OF DENTAL PLAQUE RELEVANT TO MODELS

Biofilm of linked bacterial communities:
Complex, heterogeneous, site-specific, variable, evolving, spatially organized.

Microbiota:
Bidiverse, microbial succession, interspecies structures, microcolonies, clonal blooms.

Oral environment:
Fluctuating, uncontrollable, site-specific, laminar-flow fluids (salivas, GCF).

Intraplaque environment:
Distinct, fluctuating, interacting physicochemical gradients.

Plaque growth countered by:
Host immunological/chemical defenses, fluid shear, abrasion, oral hygiene procedures.

Plaque maximum thickness (approx.)	
Smooth surface	300 µm*
Fissure	2 mm§
Approximal	5 mm¶

* Main *et al.* (1984).
§ Igarishi *et al.* (1989).
¶ Newman and Morgan (1980).

behavior and on dental pathology.

Plaque behavior and development are the outcome of a vast number of specific adherence, nutrient (Carlsson, 1997), and metabolite interactions between and among the bacteria present in the biofilm, including; micro-environment modification, synergism, competition, and antagonism (Table 2), together with interchanges between the biofilm and its environment. The ability to predict emergent properties of microbial biofilms from knowledge of the individual bacteria quickly disappears as the number of species increases beyond about two. An attempt to explain plaque behavior based on the properties of monocultures can be regarded as somewhat heroic.

Two complementary microbiological approaches have been taken to generating and studying emergent properties in biodiverse model biofilm systems. The first is construction of 'synthetic' plaque-like consortia with major plaque species (McKee *et al.*, 1985; Bradshaw *et al.*, 1989, 1990, 1993, 1994; Marsh *et al.*, 1994, 1995; Kinniment *et al.*, 1996a,b). The second is the evolution of plaque microcosms from the natural oral microflora (Sissons *et al.*, 1985, 1988a,b, 1991, 1992, 1994a,b, 1995a,b, 1996a; Tatevossian, 1988). A microcosm is "a laboratory subset of the natural system from which it originates but from which it also evolves"

TABLE 2

SOME INTERBACTERIAL INTERACTIONS MODIFYING BEHAVIOR

Environmental modifications—*e.g.*, pH, pO_2, pCO_2, Eh

Synergism—mutual adhesion
—nutrient cross-feeding
—complementation in macromolecule hydrolysis
—defense against host antibacterial factors

Competition—for adhesion, nutrients, growth factors, space

Antagonism—poisoning by metabolites, active O_2 species
—degradative enzymes
—bacteriocins

(Wimpenny, 1988). It almost always features genetic, temporal, and structural heterogeneity and may be any size—"micro" is in comparison with lakes and oceans. A microcosm after culture and evolution in an *in vitro* environment is no longer quite the natural system but rather an holistic, closely related but simpler, more controllable model.

BIOFILM TECHNOLOGIES

Model systems related to dental plaque biofilm culture models

At the boundaries of *in vitro* plaque biofilm culture systems, there are important, complementary experimental systems outside the scope of this review.

Non-biofilm culture systems. These have formed the cornerstone of oral microbiology. The most common plaque model is a batch culture of a single oral organism—which is not a biofilm, has no genotypic or spatial heterogeneity, and a changing bacterial environment far removed from that of plaque. Within these limitations, it can be a valid approach to the study of behavior relevant to that in plaque biofilms of the organism involved.

Standard chemostat mono- and co-cultures allow the environment and bacterial growth to be controlled but generally lack biofilm organization, since they need to be homogenously planktonic to function properly (Marsh, 1995b). Nevertheless, biofilms tend to form anyway, providing the basis for one set of biofilm models.

Microbial biofilm biochemical reactors. Microbial biofilms which are constructed to function in short-term experiments but are not cultured are particularly important in establishing metabolic parameters and biofilm-specific behavior for mathematical modeling (Dibdin, 1997). They usually involve biofilms with a single species of bacteria. They have included bacteria suspended in gels of defined geometry and fluid flow for the study of, *e.g.*, pH changes

(Macpherson and Dawes, 1991a,b), and cells packed onto enamel (Luoma *et al.* 1984; Assinder and Dibdin, these proceedings) or layered between membranes for the study of diffusion and metabolism rates (Dibdin, 1981; Pearce and Dibdin, 1995). Salivary sediment, the mixed salivary bacteria, many of which are clumped or adherent to epithelial cells, is partly in this category.

Intra-oral models. Intra-oral model systems include plaque growth on plastic strips (Brecx *et al.*, 1981), under bands (Boyar *et al.*, 1989), on enamel attached to teeth (Robinson *et al.* 1996; Liljemark *et al.*, 1997), and to intra-oral appliances in different configurations (*e.g.*, Nyvad and Kilian, 1987; Igarashi *et al.* 1989; Macpherson *et al.*, 1990, 1991; Pearce *et al.*, 1991). They feature mainly growth of the natural microflora in an intra-oral environment; hence they are not really artificial biofilm models. Compared with *in vivo* plaque, these models give increased access and sampling possibilities and do not put host tissues at risk; those on removable appliances allow for experimental treatments outside the mouth, but they suffer other difficulties of natural plaque studies (Nyvad, 1993; Zero, 1995; Marsh, 1995a).

Some ingenious hybrid systems exist. One intra-oral model involves pre-formed cariogenic biofilms of *Streptococcus mutans* between enamel blocks in a palatal appliance (Zero *et al.*, 1992). Changes in cariogenic attack on enamel with increasing plaque depth, affected by the amount of extracellular polysaccharide present, and hence cell packing, have been demonstrated (Zero *et al.*, 1996). In another model, bacterial monolayers are applied to the enamel, followed by growth of the natural microflora (Liljemark *et al.*, 1988). The finding in this system that growth of natural plaque was unaffected by variations in the adherence properties of initial streptococcal monolayers (Skopek *et al.*, 1993) is of particular significance to the issue of how initial colonizing bacteria affect the development of mature plaque. Such models both benefit and suffer from being sited in the natural but site-specific, uncontrolled oral environment.

Major oral biofilm culture system technologies in current use

Tatevossian (1988) has comprehensively reviewed culture systems for plaque biofilms and their development up to the mid-1980s. I will focus on more recent developments.

Chemostat-based systems. Experimental surfaces submerged in chemostats (Keevil *et al.*, 1987; Li and Bowden, 1994a,b, 1996; Bowden and Li, 1997; Burne *et al*, 1997; Marsh and Bradshaw, 1997) or in flow cells supplied by chemostats (Herles *et al.*, 1994; Allison *et al.*, 1997) have been developed into a set of powerful model systems. Since they are mainly described elsewhere in these symposium proceedings, I will not do so in detail.

Growth-rate-controlled biofilm fermenter (GRBF). This gives controlled media-limited outgrowth rates of thin filter-deposited biofilms, analogously to the chemostat (Gilbert *et al.*, 1989, 1997; Gilbert and Allison, 1993). Analysis of steady-state biofilms and the effluent which contains a cohort of freshly divided daughter cells allows for investigation of the growth-rate dependence and cell-cycle specificity of anti-bacterial agents.

'Artificial mouths'. Pigman *et al.* (1952) coined the term 'artificial mouth', applied subsequently to similar plaque biofilm culture systems. Most of the early studies focused on dental caries rather than on the plaque biofilm itself, sometimes with insufficient attention to sterility.

Several major current designs of artificial mouths were developed in the 1970s. Russell and Coulter (1975) developed a culture system which included sectioned teeth, pH, and Eh electrodes. They studied the plaque microbiology with an emphasis on pH and Eh responses and effluent analysis of microcosm, monoculture, and up to four species consortia (Russell and Coulter, 1975, 1977; Ahmed and Russell, 1978; Russell and Tagg, 1981). Dibdin *et al.* (1976) developed an artificial mouth with up to six replicated chambers for independent controlled plaque growth and control of nutrients by pumps. They made preliminary studies of microcosm plaques. These culture systems were combined by Donoghue and colleagues using separate culture chambers (Hudson *et al.*, 1986), and by us, as described below, to develop a multiplaque single-chamber culture system (Sissons *et al.*, 1991, 1992; Wong *et al.*, 1994).

Another artificial mouth lineage involves rotating enamel blocks or hydroxyapatite disks under a nutrient fluid supply. In one example, the Orofax, slowly melted frozen saliva dripped onto hydroxyapatite disks mounted between slides in a slide projector carousel. It had problems with reproducible growth but produced microcosm plaques, Gram-stained smears of which were similar to natural plaques (Yaari and Bibby, 1976; Bibby and Huang, 1980). The major system in current use, developed by Noorda *et al.* (1985), consists of a computer-controlled fluid supply and rotating platter which contains a series of enamel blocks or hydroxyapatite disks enclosed in a fermenter and is inoculated from batch culture. Mono- (Noorda *et al.*, 1986a,b), di-, and tri-culture (Cummins *et al.*, 1992; Simmonds *et al.*, 1995) consortia have been studied.

The constant-depth film fermenter (CDFF). This culture system limits biofilm thickness. Biofilms are grown in a set of 15 pans, each containing 5 or 6 disc depressions of pre-set depth, usually 300 μm, and when biofilm growth reaches the top, it is limited by sweeping of the surface with a Teflon blade. Fluids are also delivered by this blade (Peters and Wimpenny, 1988; Wimpenny *et al.*, 1993; Wimpenny, 1997). Until this maximum depth is reached, there is a planktonic culture above the biofilm, forming a second phase. The CDFF used for plaque studies can be regarded as a type of artificial mouth. It is now being operated as a two-stage system, with its inocula established as a steady-state consortium in an anaerobic chemostat with aerobic CDFF biofilm growth (Kinniment *et al.*, 1996a; see below).

The multiplaque artificial mouth. This is a fairly generic

artificial mouth which has developed into a flexible system allowing for a range of experimental regimes and modifications.

Current design features include:

- long-term independent growth of five replicate plaques from the same inoculum, at the same temperature in the same gas phase (Sissons *et al.*, 1991);
- computer control of (i) three or more independent fluid lines to each plaque for simulated oral fluid, carbohydrate, and experimental treatments, and (ii) data acquisition from micro-pH and reference electrodes, allowing for continuous pH measurement for up to two weeks (Sissons *et al.*, 1992; Wong *et al.*, 1994); and
- a wide choice of possible plaque growth supports and substrata, including tooth tissues (Sissons *et al.*, 1992; Cutress *et al.*, 1995; Shu *et al.*, 1996) [The standard plaque support system consists of a 2.5-cm-diameter autoclavable Themanox (TM) tissue-culture coverslip to give radially symmetrical plaques which are positioned on a glass ring with posts to standardize fluid dynamics. Plaque and coverslip can be weighed during growth so that plaque wet-weight can be measured (Sissons *et al.*, 1995a). As well as direct plaque sampling with a loop or spatula, wedge samples of coverslips with undisturbed plaque can be removed and processed for electron microscopy. Up to six blocks of enamel, dentin, or sectioned teeth can be embedded in a 2.2-cm-diameter disk of resin for the measurement of cariogenic attack by hardness testing or microradiography (Shu *et al.*, 1996).]

In the standard growth protocols, a nutritional analogue of saliva, BMM (Table 3), based on the medium of Glenister *et al.* (1988), is supplied at 3.6 mL *per* hour *per* plaque. Every 6 or 8 hours, 1.5 mL of 5 or 10% sucrose is supplied for 6 min to mimic meals. All of these parameters can be varied.

TABLE 3

ORAL FLUID ANALOGUE (BMM) USED IN 'MULTIPLAQUE ARTIFICIAL MOUTH' MICROCOSM PLAQUE STUDIES (Sissons *et al.*, 1991)

Component	Concentration
Trypticase peptone	0.5 %
Proteose peptone	1.0 %
Yeast extract	0.5 %
KCl	0.25%
Part purified pig gastric mucin	0.25%
Hemin	5 mg/L
Menadione	1 mg/L
Urea	1 mmol/L
Arginine	1 mmol/L

Comparison of plaque biofilm culture technologies

Different biofilm culture technologies have distinctive features and approaches to modeling the oral environment, as summarized in Table 4.

In chemostats, there is a controlled fluid environment and a planktonic phase with a nutritionally limited growth-rate, which interacts biologically with the biofilm. Potentially, a large number of replicate samples and a variety of surfaces can be used, but plaques grown under different conditions require separate chemostats. Removing the surfaces outside the chemostats to give external biofilm growth increases flexibility of sample configuration, fluid dynamics, plaque monitoring possibilities, and experimental treatments. The main differences between the growth-rate-controlled biofilm fermenter and chemostat-related systems are the former's lack of intervening planktonic phase, fluid flow through the biofilm instead of across its surface as in mucosal but not hard-surface biofilms, possibilities for cell-cycle analysis, but limited sample replication and choice of substratum.

Artificial mouths tend to feature independently grown plaques with essentially unrestrained growth, being limited in size, to a small degree, by liquid shear. Compared with chemostat systems, they lack significant planktonic phases and are less controlled by the physical properties of the oral fluids which are, however, applied with approximately laminar flow as in the oral cavity, instead of turbulent flow. They have flexible and more precisely timed fluid application regimes, properties such as pH can be controlled by manipulation of the supply of appropriate substrates (see below), and nutrient cycling with oral fluid to mimic meals is usually present. They can grow a number of plaques from replicate inocula under different controlled nutrient conditions. Because of unrestrained growth, exact sample replication within each plaque is limited, except with the controlled-depth film fermenter, but subsampling and effluent analysis are usually possible. With our standard plaque supports in the artificial mouth, plaque wet-weight accumulation can be measured (Sissons *et al.*, 1995a). Growth of mature plaques over weeks can be studied. Treatments with semi-solids such as toothpaste can be applied outside the culture chamber (Zampatti *et al.*, 1994). Long-term continuous monitoring of biofilm pH behavior by electrodes is a major advantage (Russell and Coulter, 1975; Hudson *et al.*, 1986; Sissons *et al.*, 1992; Wong *et al.*, 1994). This is not easily possible in the current-configuration constant-depth film fermenter, which has traded this facility for constant geometry plaques and a large number of sample replicates (Peters and Wimpenny, 1988; Wimpenny *et al.*, 1993).

Overall, each culture system has strengths, limitations, and difficulties, with no one system better than the others for all purposes. Hybrid systems may focus most effectively on specific problems. Key issues include available expertise and technologies which can be moderately complicated, focus on the number of different growth conditions required and on plaque morphology, flexibility of experimental protocols, number of replicates and types of samples, possibilities for biofilm and substratum analysis, and modeling of surface (and pellicle), fluid, and gaseous environments. Separate choices need to be made of culture technology and microbial

TABLE 4

DISTINCTIVE FEATURES OF BIOFILM CULTURE TECHNOLOGIES

Parameter	GRBF	Chemostat Surfaces	CDFF	Artificial Mouths
Duration	hours to days	hours to days	days to weeks	days to weeks
Planktonic phase	none	controlled	[none]	none
Growth control by media	direct	*via* planktonic phase	yes	yes
Fluid flow	vertical	turbulent/laminar*	laminar	laminar
Shear force limitation	none	fluid	mechanical	none
Defined thickness	no	no	yes	no
Timed CHO/reagents	(pulse?)	pulse	yes	yes
Control of biofilm pH	media?	*via* planktonic phase	metabolic	metabolic
Electrode monitoring	no	possible	no	yes
Alternative substrata	no	yes	some	yes
Independent regimes	1	1(+)	1	≈ 6
Plaque replication	low	high	high	≈ 6
Plaque subsampling during growth	no?	possible	no	yes

* Laminar flow is possible with external surfaces.

system. The same technologies are generally applicable to consortia and microcosm systems.

BIODIVERSE PLAQUE BIOFILM MODEL SYSTEMS

Biofilm consortia of up to 6 oral bacteria

Until recently, published biofilm studies of oral bacteria consortia extended to 4 or 5 species. Russell and colleagues showed in di-cultures that sucrose and extracellular polysaccharide formation were important in biofilm production (Russell and Coulter, 1977), that *Actinomyces naeslundii* was cleared from biofilms of *S. mutans* and *S. sanguis* (Ahmed and Russell, 1978), and that bacteriocin antagonism between *Streptococcus salivarius* and *S. sanguis* occurred (Russell and Tagg, 1981).

Donoghue and co-workers, in an elegant series of studies, showed that *Streptococcus rattus* and *Streptococcus oralis* co-culture biofilms were affected by carbohydrate supply (Donoghue *et al.*, 1980, 1983), inoculation sequence (Donoghue *et al.*, 1983), and antagonism mediated by H_2O_2 and the lactoperoxidase system (Donoghue *et al.*, 1985, 1987; Donoghue and Perrons, 1988a). A consortium including *S. mutans, S. oralis, A. naeslundii*, and *Veillonella dispar* showed significant cross-feeding, and *S. mutans* antagonized the *A. naeslundii* (Donoghue and Perrons, 1988b; Perrons and Donoghue, 1990). Considerable but not complete colonization resistance developed against *S. mutans* in 24-hour plaques. The ability of *S. mutans* to colonize was unaffected in a glucan-deficient non-cariogenic mutant (Donoghue and Perrons, 1991). Recently, the Noorda/Purdell-Lewis system has been used to demonstrate that the bacteriocin Zoocin A from *Streptococcus zooepidemicus* reduces by about 2 logs the proportion of *S. mutans* in a tri-culture plaque with *S. sanguis* and *A. viscosus* (Simmonds *et al.*, 1995).

We have also been studying four-species-consortia biofilms in the artificial mouth using the putative root caries pathogens: *S. mutans, Streptococcus sobrinus, Lactobacillus rhamnosus*, and *A. naeslundii*. Reasonably reproducible four-bacteria biofilm consortia were formed after 11 days, even with sucrose changed to glucose as carbohydrate supply (Shu *et al.*, 1996). The resting pH of these consortia was low (about 5.2 to 5.8), and probably, as a result, *A. naeslundii* had trouble remaining established, even if inoculated first. It ranged up to 4% in some experiments. These four-species consortia caused caries-like lesions on bovine enamel, dentin slabs, and sectioned human teeth, and a greater pH decrease and enamel softening than the monoculture biofilms. In a six-species consortium (Table 5), fluoride continuously applied at 5 and 20 ppm decreased *S. mutans* but not *S. sobrinus*, replaced mainly by *L. rhamnosus*. The cariogenic attack reflected by enamel softening was reduced by the F treatments, similarly to microcosm plaques (Cutress *et al.*, 1995).

Synthetic plaque-like consortia of 9 (plus) oral bacteria

Development of nine- to 11-member-consortia biofilm systems by Marsh and colleagues is a major advance in biodiverse synthetic plaque models (McKee *et al.*, 1985). These systems contain representatives of major plaque species (Table 6) selected to allow for complete microflora analysis. In anaerobic chemostats, steady-state consortia are formed containing all species, maintained by co-operative

TABLE 5

EFFECT OF CONTINUOUSLY SUPPLIED FLUORIDE ON THE COMPOSITION AND CARIOGENICITY OF SIX-MEMBER CARIES CONSORTIA PLAQUES (Shu *et al.*, 1996)

Parameter	F Concentration 0 ppm	5 ppm	20 ppm
Composition (%)*			
Streptococcus mutans ATCC 25175	68	26	5
Streptococcus sobrinus O1H1	14	24	20
Streptococcus mitis SK146	2	20	0
Actinomyces naeslundii HNG 243	0	1	0
Veillonella dispar ATCC 12745	10	12	9
Lactobacillus rhamnosus ATCC 7461	8	16	86
Enamel Softening (μm)§	72.2	49.5[a]	23.5[b]

* Half the entire plaque was analyzed by spiral plating of 50-fold dilutions in triplicate Brain-Heart-Infusion-Yeast Extract agar, and in duplicate on MS, MSB, CFAT, Rogosa, and Veillonella agars (Slots, 1986).

§ There were four enamel blocks *per* plaque growth station, each with six paired before and after treatment measurements of enamel microhardness by Knoop diamond indentation. Softening was estimated as the increase in indentation length; the control indentations were *ca.* 49 μm.

[a] $p < 0.05$.

[b] $p < 0.0001$.

glycoprotein degradation (McDermid *et al.*, 1986; Bradshaw *et al.*, 1994). Planktonic chemostat versions of these consortia have been used to show that low pH rather than carbohydrate availability favors *S. mutans* (Bradshaw *et al.*, 1989) except in 1 mmol/L fluoride, where pH-uncontrolled cultures pulsed with glucose lost *S. mutans* and correspondingly favored *Lactobacillus casei* (Bradshaw *et al.*, 1990). They have also been used to investigate the action of antimicrobials (McDermid *et al.*, 1987; Marsh, 1991b, 1992, 1993; Bradshaw *et al.*, 1993).

Consortium biofilms containing all species have been cultured on hydroxyapatite (HA) surfaces suspended in the chemostat and showed effects similar to those of glucose/pH pulsing (Marsh *et al.*, 1994). Conditioning the HA surface with saliva or glucosyltransferase preparations differentially affected both total plaque growth and individual species (Marsh *et al.*, 1994; Bradshaw *et al.*, 1995). Differences between planktonic and biofilm phases in response to pyrophosphate and a synergistic inhibition of biofilm growth by combinations of triclosan with pyrophosphate or zinc citrate were demonstrated (Marsh and Bradshaw, 1993). With a first-stage anaerobic chemostat feeding an aerobic chemostat containing HA surfaces, again all species grew in the biofilm (Marsh *et al.*, 1995). Initially, *Neisseria subflava* predominated, but later, obligate anaerobes did so even if *N. subflava* was omitted. The anaerobes, even in an aerated planktonic phase, were protected from O_2 by co-aggregation with aerobic and facultative species, mediated by *Fusobacterium nucleatum* for species pairs which did not otherwise co-aggregate (Bradshaw *et al.*, 1996, 1997). Overall, these studies reinforce the potential usefulness of the chemostat-based plaque consortium system in biofilm studies.

TABLE 6

THE 'MARSH' CONSORTIA BACTERIA

1	*	*Streptococcus mutans* R9
2		*Streptococcus sobrinus* ATCC 227351
3	*	*Streptococcus gordonii* NCTC 7865
4	*	*Streptococcus oralis* EF 186
5	*	*Actinomyces naeslundii* WVU 627
6	*	*Lactobacillus casei* AC 413
7	*	*Neisseria subflava* A1078
8	*	*Veillonella dispar* ATCC 17748
9	*	*Porphyromonas gingivalis* W50
10		*Prevotella nigrescens* T588
11	*	*Fusobacterium nucleatum* ATCC 10953

* In nine-bacteria biofilm consortia.

The above studies have also laid the basis for development of consortia biofilms in an aerobic constant-depth film fermenter after inoculation over 8 hr with a steady-state anaerobic chemostat consortium (Wimpenny, 1995; Kinniment *et al.*, 1996a). Successive increases in *S. mutans*, *L. casei*, and *P. gingivalis* occurred, with decreases after the first few days in *S. oralis*, *V. dispar*, and *A. viscosus*, to yield a relatively constant composition after about 20 days of growth, possibly a *quasi*-steady state. There are difficulties with the concept of an actual steady state in complex-structured biofilms. Microscopy showed distinct layers: surface *Neisseria*, fusobacteria in inner layers, and moribund cells in the basal layers. The final composition of the microflora varied between runs, suggesting that more than one set of bacterial communities can be stable in one set of environmental conditions. Accumulation of mutations during growth was suggested as a possible source of variation. Pulses of chlorhexidine induced species-specific changes consistent with clinical findings (Kinniment *et al.*, 1996b). Overall, these consortia systems embody a powerful synthetic approach for studying the behavior of plaque biofilms.

Plaque microcosms evolved from the natural microflora

Microcosm biofilms have proved to be powerful, relevant, and useful experimental systems in many areas of environmental microbiology and microbial ecology (Wimpenny, 1988, 1995, 1997). Because they originate directly from the whole-mixed natural microflora, microcosms embody its complexity and heterogeneity of genotype, and biofilm microcosms develop spatial heterogeneity and structure. They evolve and hence are not

TABLE 7

GRAM-STAIN MORPHOTYPES, UREOLYTIC FLORA, AND UREASE LEVELS OF 'DIBDIN' SINGLE-CHAMBER CULTURE SYSTEM ARTIFICIAL MOUTH PLAQUES (after Sissons *et al.*, 1988a)*

Saliva Origin§	EH				CS					
Enamel or Chamber Wall¶	E	E	E	W	E	E	W	E	W	
Plaques in the Same Run¶										
Ratio: Gram + ve/ - ve	2.6	1.8	4.0	4.0	0.92	1.7	0.92	32	13	
Rods %	12	30	13	10	36	15	25	2	2	
Ureolytic Bacteria %	35.1	13.5	25.0	11.6	21.1	19.6	8.8	63.3	31.7	$r^2 = 86\%$#
Plaque Urease Level (nmol NH_3/min/mg protein)	431	275	342	279	332	362	132	994	697	

* Samples were taken at the same time for urease and protein measurement and for cultural analysis. Sixty random colonies from brain-heart-infusion yeast extract agar plates were subcultured, purified, Gram-stained, and tested for ureolytic activity on urea-segregation agar.

§ Plaque-enriched saliva from single individuals, EH and CS, was cultured.

¶ Location of plaque on a suspended block of tooth enamel (E) or growing periodically submerged on the culture chamber wall (W). Corresponding plaques in the same culture vessel taken at the same time are indicated by the line.

r^2 was derived by linear regression of the percentage ureolytic bacteria detected against the plaque urease level.

steady-state systems, in that they resemble plaque *in vivo*.

In dental research, some early culture systems were, in essence, plaque microcosms (Tatevossian, 1988), but interest in this approach and belief in its value seem to have waned. Reasons probably include the intrinsic variability and ill-defined nature of the inoculum, the ill-defined complex heterogeneity of the resulting microcosm plaques, and reproducibility issues. A suggestion that the transient organisms present in saliva or supragingival plaque outgrew the normal microflora (Sidaway *et al.*, 1964) is probably also a factor. Insufficient appreciation of the significance of a highly biodiverse microflora and the 'biofilm lifestyle' (Bowden and Li, 1997; Caldwell *et al.*, 1997) may have also played a role. Developments in ecological theory on the role of the biodiversity (Naeem *et al.*, 1994; Steneck and Dethier, 1994; Tilman and Downing, 1994; Liljemark *et al.*, 1997) of *in vitro* technologies, together with existing findings in plaque microcosms, suggest that re-assessment of their potential is warranted.

Prior to the 1980s, little useful information was obtained from microcosm plaque studies beyond the demonstration that plaque and saliva inocula give plaque-like structures (Dibdin *et al.*, 1976), even in batch enrichment cultures (Leadbetter and Holt, 1976), and attack enamel (*e.g.*, Pigman *et al.*, 1952; see Tatevossian, 1988). Singer and Buckner (1980) showed that a plaque-inoculated artificial mouth microcosm elaborated a range of cell toxins. A key study (Marsh *et al.*, 1983) of chemostat microcosms inoculated from plaque showed that a complex mixed microflora was established with a splash zone biofilm which, under glucose limitation, contained spirochetes which are indicative of considerable cross-feeding in a mature, possibly periodontopathic microflora (ter Steeg and van der Hoeven, 1990). In the late 1980s, it was shown that inclusion of acrylic tile surfaces generated complex biofilms (Keevil *et al.*, 1987) and that mucin in a version of BMM medium enhanced the biodiversity of a planktonic microcosm (Glenister *et al.*, 1988). Subgingival plaque chemostat enrichments in serum, to simulate gingival crevicular fluid, generated a succession of periodonto-pathogens, also including spirochetes, with directly demonstrated extensive cross-feeding and complementation of glycoprotein degradation (ter Steeg *et al.*, 1988; ter Steeg and van der Hoeven, 1989, 1990). These important studies demonstrate the potential for the development of realistic subgingival plaque microcosms.

My initial studies on artificial mouth plaques in the 1980s (Sissons *et al.*, 1985, 1988a,b; Sissons and Cutress, 1987; Pearce and Sissons, 1987) used a single-chamber variant of the Dibdin system, growing a plaque from saliva on an enamel block supplied with 1% peptone-0.5% yeast extract continuously and with sucrose every 4 hrs. This protocol extensively softened enamel (unpublished). Analysis of plaque urea metabolism rates, Gram-stain morphotypes, and isolated ureolytic microflora (Table 7) showed that successive microcosms inoculated from the same person differed greatly in both microflora composition and urea metabolism rate (Sissons *et al.*, 1988a). To get reproducible microcosm plaques, it is clearly essential that one use the same inoculum for each plaque. Plaques grown on the tooth and culture vessel wall, although under somewhat different nutrient regimes; were quite similar in characteristics, demonstrating some robustness of composition to variation in environment. The microflora percentage of the isolated

ureolytic bacteria (Sissons *et al.*,1988a,b) almost entirely explained (r^2, 86%) the ureolysis rate of the plaques, indicating considerable environmental standardization of the system between runs with respect to the level of urease, an environmentally regulated enzyme (Sissons *et al.*, 1990, 1995c; Mobley *et al.*, 1995).

Microcosm plaques in the multiple artificial mouth

Various features of microcosm plaques which we have studied in the multiple artificial mouth reinforce the evidence that they are realistic and useful plaque models.

Biodiversity and selective processes in the evolution of microcosms. We normally initiate microcosm plaques from plaque-enriched saliva rather than plaque, with repeat inoculation on d3 and d5 of growth to encourage acquisition of climax species. As well as laying a pellicle, the rationale for the use of saliva is grounded in unresolved issues about biodiversity, the role of natural selection in evolving microcosms, and the possibility that accessible smooth-surface plaque may not contain founder species or climax species for other areas of the dentition. By contrast, plaque-enriched saliva contains micro-organisms pooled from all ecosystems in the oral cavity (Sissons *et al.*, 1989). A fundamental question is to what degree the environment selects for particular species and programs a specific succession of organisms from a more biodiverse pool, such as plaque-enriched saliva. It is likely that selective pressures will be directed to plaque function and only indirectly to the composition of the microflora, supported by the run-to-run variation found in CDFF nine-species-consortia biofilms (Kinniment *et al.*, 1996a). The concept of convergent evolution of different composition but equivalent function ecosystems has received recent support from studies in marine algal communities showing extensive changes in dominant algal species but constant predator resistance and rates of nutrient consumption (Steneck and Dethier, 1994). This concept may prove helpful in the understanding of dental disease. Plaque ecology induces dental pathology: Caries, gingivitis, and periodontitis result from ecological shifts in the biodiverse plaque microflora (Newman, 1990; Bowden, 1991; Marsh and Martin, 1992; Marsh, 1995a; Marsh and Bradshaw, 1997). This concept is encapsulated as the 'ecological plaque hypothesis' (Marsh, 1995a; Marsh and Bradshaw, 1997) and reinforces the need for caution in extrapolation from studies of single pathogens such as *S. mutans* or *P. gingivalis* to dental pathology [the traditional medical approach to individual pathogens involving Koch's criteria is inapplicable to biofilms of the normal body microflora (Caldwell *et al.*, 1997)].

Composition and structure. The composition of saliva-evolved microcosm plaques reflects a mainly anaerobic, substantially Gram-negative microflora, consistent with natural supragingival plaques and chemostat plaque enrichments (Table 8; Sissons *et al.*, 1991). We cannot rule out overgrowth by transient organisms but have looked quite thoroughly and did not find staphylococci or yeasts. Plaque protein is about 5% of the wet weight (Sissons *et al.*, 1995a), typical of relatively unpacked plaques (Gilmour *et al.*, 1979), and electron microscopy shows a diverse morphology microflora. We have seen flagellated curved rods and, with serum supplementation, spirochetes. Motile bacteria are present, sometimes in considerable numbers. Microcolonies, ordered associations, various stages of degrading bacteria, and complex extracellular structures occur (Pearce *et al.*, 1991; Sissons *et al.*, 1991). Overall, electron microscopy shows a biofilm consistent with natural plaques.

TABLE 8

CULTURAL ANALYSIS OF MICROCOSM PLAQUE*

	Media§	% of Anaerobes
Total colony-forming units		
Anaerobic	TSB	100
Gram-negative		43
Rods		30
10% CO_2 in air	TSB	27
Selective media		
10% CO_2 in air:		
Haemophilus sp.	HMC	0.0005
Neisseria sp.	TSV	ND#
A. a.¶	MGB	ND
Yeasts	Sabouraud	ND
Staphylococci	Staph110	0#
	MN	0
Anaerobic (10% H_2, 10% CO_2, 80% N_2)		
Streptococci	MS	37
Mutans sp.	MSB	8
Fusobacteria	CVE	3
Veillonella sp.	V	6
Lactobacillus sp.	Rogosa	13
Actinomyces sp.	CFAT	8

* The plaque was grown for 18 days with BMM with the yeast extract replaced by CVA vitamin enrichment (Life Technologies Inc., NY). Ten bacteriological loopfuls were taken from representative areas of the plaque, combined, and dispersed by vortex mixing with glass beads. Fifty-fold dilutions were spiral-plate-cultured on the media shown.

§ TSB is tryptic soy agar enriched with 1 mg/L menadione, 5 mg/L hemin, and 10% defibrinated sheep blood (Life Technologies). MN is mannitol salt agar, MS(B) is Mitis Salivarius (bacitracin) agar, and other media are as in Slots (1986).

¶ *A.a.* is *Actinobacillus actinomycetemcomitans.*

\# ND = not detected among colonies present in highest measurable dilution; 0 = not present in original plaque suspension.

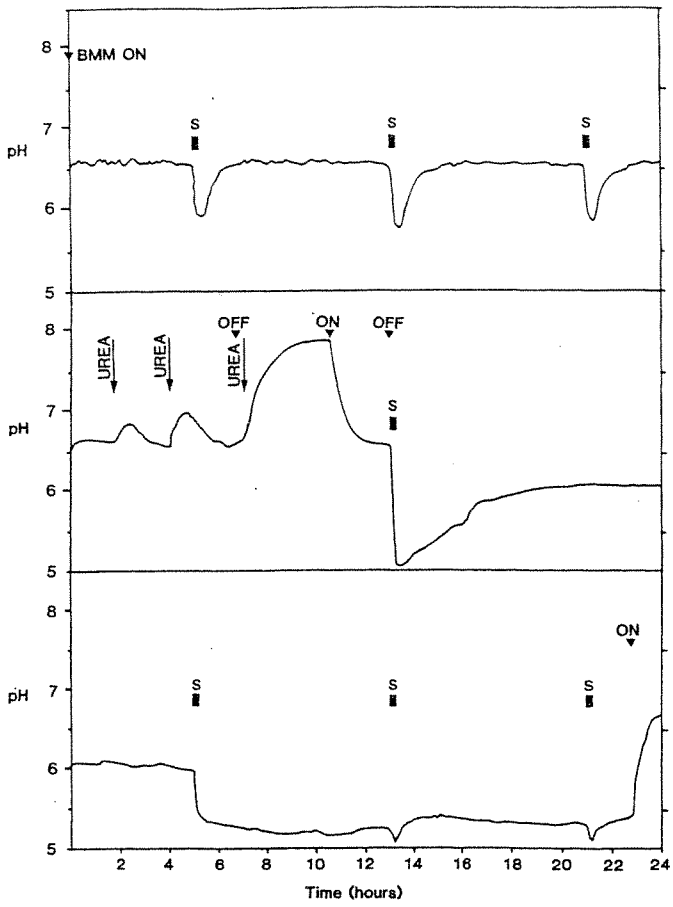

Fig. 1—Effect on microcosm plaque pH of consecutive sucrose and urea applications over 3 days with and without a flow of simulated oral fluid (BMM, Table 3). S, sucrose; UREA, an urea application for 6 min (1.5 mL). ON and OFF indicate changes in BMM flow.

Plaque growth and reproducibility. Large plaques develop which, after several weeks' growth, reach a depth of several mm, weigh 1 to 2 g, and model the extremes of thick, sheltered approximal plaques (Newman and Morgan, 1980; Sissons *et al.*, 1992, 1994a,b). Plaque growth is mainly limited by the coverslip area. Excess plaque falls from the holder to the base of the chamber, where a continuous thick biofilm develops which helps remove oxygen from the chamber. We have grown plaques for 7 weeks, a six-month time has been reported (Sidaway *et al.*, 1964), and there seems to be no reason why they cannot grow indefinitely. The rate of plaque accumulation under standard conditions declined over the first five days of culture, with doubling times of from 3 to 7 hrs over the first day of growth, from 9 to 21 hrs over the second day, and subsequently from 20 to 40-plus hrs (Sissons *et al.*, 1995a). These declining growth rates are the same as those for supragingival plaque *in vivo* (Weiger *et al.*, 1995). Thus, the large plaque sizes finally reached are a consequence of limited mechanisms to counter plaque accumulation rather than unnatural growth rates. The relative importance of different mechanisms limiting plaque accumulation *in vivo* (Table 1) is site-specific and uncertain.

Sometimes there was biphasic growth with second-stage linear growth (Sissons *et al.*, 1995a). In one run, one plaque maintained its high initial growth rate to give a yellow plaque bloom containing a mixed microflora. Because of spatial differentiation and extensive microcolony formation, plaque growth can perhaps be regarded as a series of blooms (Skopek *et al.*, 1993) which are usually restricted by competition and antagonism with the rest of the microflora. Stochastic processes undoubtedly play a part in developing variability of composition during growth. For this reason, a quest for growing reproducible-composition microcosm biofilms may be futile, especially in plaques grown for long periods. It should probably be replaced by a search for reproducible-function plaques. Functionally reproducible plaques may be attainable, since we have found the resting pH to be highly reproducible (Sissons *et al.*, 1991, 1993, 1996b) and have established a coefficient of variation for within runs of about 25% for plaque accumulation (Sissons *et al.*, 1995a) and urease levels (Sissons *et al.*, 1995b).

Plaque pH and responses to sucrose and urea. pH responses to applications of sucrose and 500 mmol/L urea in microcosm plaques correspond to those expected in natural plaque (Fig. 1). Stopping the oral fluid flow enhanced pH responses; restoring it was necessary for a return to the resting pH. The effects of even a slow oral fluid flow were profound (Fig. 1; Sissons *et al.*, 1992, 1994a,b), as predicted by the Dawes/Dibdin mathematical models (Dawes, 1989; Dibdin, 1990a,b, 1995, 1997). Intraplaque pH gradients have been found after sucrose application; there were Stephan pH curves on the plaque surface but virtually no pH response in inner layers (Sissons *et al.*, 1992). Urea generated pH gradients of opposite polarity which were stable for many hours in the absence of a BMM flow (Sissons *et al.*, 1994b). With BMM flowing, pH gradients changing in polarity were formed, with the highest pH reached in mid-plaque (Fig. 2 and Sissons *et al.*, 1994b). Hence, the pH of structured, diffusion-limited biofilms involves a delicate interaction of oral fluid flow with the supply, clearance, and metabolism of substrates, buffers, and products involving dynamically reversing intraplaque pH gradients.

Significant levels of urea occur in saliva (Kopstein and Wrong, 1977; Macpherson and Dawes, 1991a) and crevicular fluid (Golub *et al.*, 1971) and may be important in modifying plaque pH and dental pathology (Peterson *et al.*, 1985). The resting pH of microcosm plaques in BMM containing no urea is reliably pH 6.4 ± 0.1, regardless of inoculum source (Sissons *et al.*, 1991, 1993, 1996b). This is apparently an example of evolution to convergent function in biofilms in a particular *in vitro* nutrient and flow environment. Supplementing the BMM with 20 mmol/L urea raised the resting pH above pH 7 (Fig. 3) and gave sucrose-induced pH responses reminiscent of Stephan's (1944) pH curves associated with caries activity (Sissons *et al.*, 1993, 1996b). Probably, the plaque microbial composition present is both determined by and interacts with its particular fluid and nutrient environment to 'set' the resting pH. An ability to

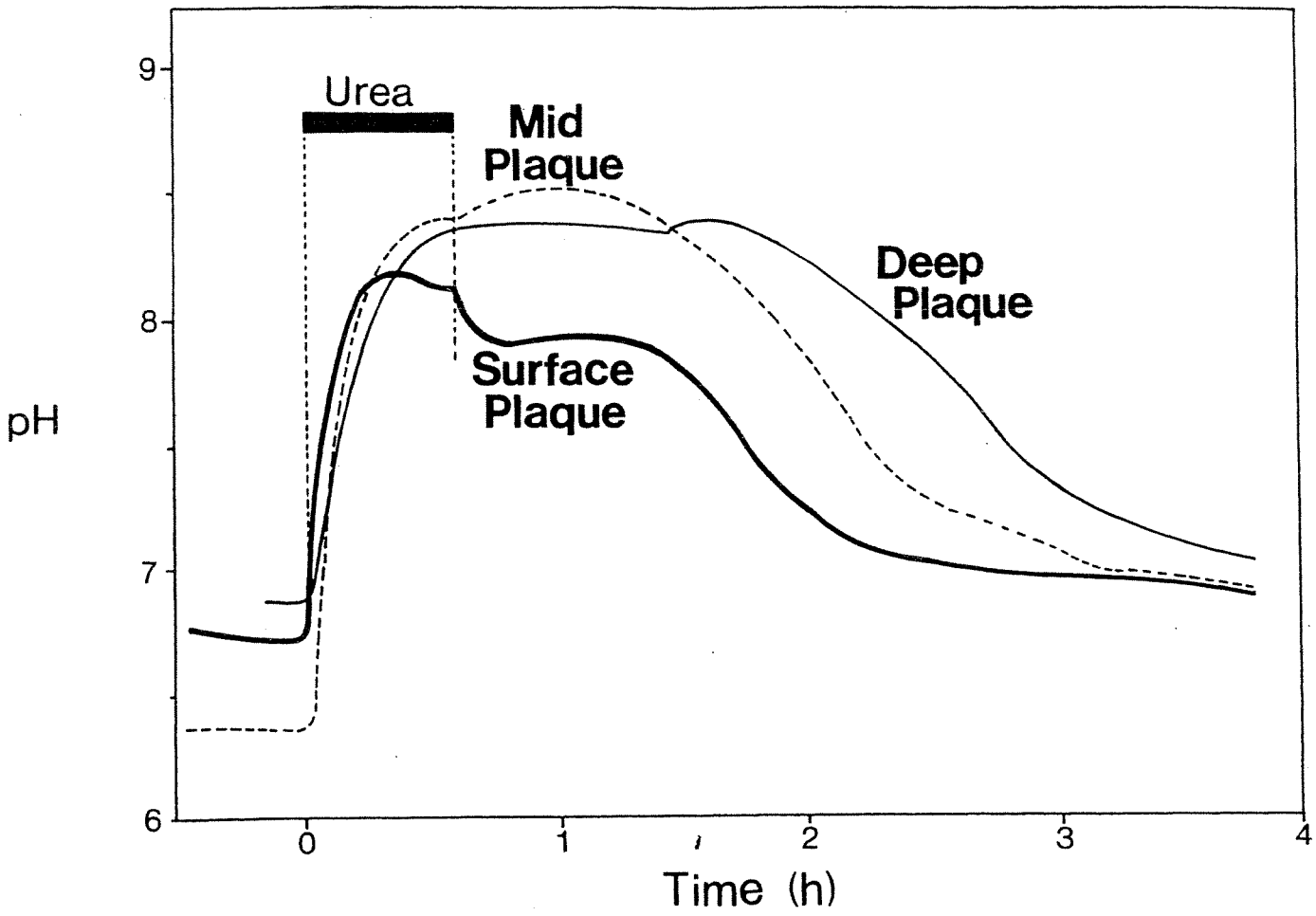

Fig. 2—Plaque pH gradients induced by 30-minute applications of 500 mmol/L urea; initially alkaline outside, then progressively reversing to become alkaline inside. Sequential pH responses to applications of urea were measured with the pH electrode at the surface, mid-point, and base of a convex 6 mm maximum depth plaque (after Sissons et al., *1994b).*

manipulate the plaque pH range metabolically through controlling the urea supply allows for experiments on the pH dependency of biofilm processes and dental pathology.

Regulation of plaque ureases. The control of the macro-environment which is possible in biofilm microcosms makes it possible directly to address environmental regulation issues which are impossible to study *in vivo.* An example is control by effectors of urease levels in plaque. Manipulation of urea, arginine, and ammonia levels showed that urea up-regulation but destabilization, arginine and ammonia down-regulation, and possibly stabilization of plaque urease levels occur in a complex time-dependent fashion (Sissons *et al.*, 1995b).

Plaque mineralization. Plaque microcosm biofilms have special advantages for the study of plaque processes where the identity of the microbes involved is uncertain, the significance of various possible processes involved is unknown, and the relevant events are buried in the biofilm. A particularly important example is mineralization of plaque to calculus, a biofilm-controlled interaction between a range of strain- not species-specific bacterial activities and the localized intraplaque environment, where the calcium phosphate supersaturation fluctuates with pH (Fig. 4).

Microcosm plaques have been mineralized by a procedure based on a pH rise induced by 500 mmol/L urea present in a pH 5, 20 mmol/L calcium, 12 mmol/L phosphate solution also containing 5 mmol/L monofluorophosphate (Pearce and Sissons, 1987; Pearce *et al.*, 1991; Sissons *et al.*, 1991, 1995c) which deposits fluorhydroxyapatite into plaque *in vivo* (Pearce, 1981, 1984). With plaque of resting pH of 6.3-6.5, this solution, applied 6 min every 2 hrs for 10 days, increases the Ca levels over 20-fold to about 2-3 nmol/mg plaque protein (Table 9; Sissons *et al.*, 1991, 1995c). Raising the resting pH by supplementing the BMM with urea increased mineral deposition and the Ca:Pi ratio, the latter perhaps indicating some carbonate as well as apatite formation. Mineral levels in 20 mmol/L urea reached about 35% of the dry weight. Hence, the plaque pH range had a major effect on mineralization, even by this procedure, which elevates the pH above 8 during mineral deposition (Wong *et al.*, 1996).

TABLE 9

MINERALIZATION OF MICROCOSM PLAQUES BY CPMU*—EFFECT OF RAISING THE PLAQUE RESTING pH BY SUPPLEMENTING THE BMM WITH UREA (Sissons *et al.*, 1995c; Wong *et al.*, 1996)

BMM Supplement (mmol/L)	Resting pH	Ca	Pi	F	Ca:Pi Ratio	F:Ca Ratio x1000
		(mmol/g plaque protein)				
None	6.5	3.4	1.9	0.13	1.81	38
Urea - 5	6.7	5.2	2.1	0.20	2.49	38
Urea - 20	7.2	14.3	5.8	0.35	2.46	25

* CPMU—20 mmol/L Ca, 12 mmol/L Pi (phosphate), 5 mmol/L monofluorophosphate, 500 mmol/L urea at pH 5.0—was applied to 21-day-old plaques for 6 min every 2 hrs for a further 10 days. Plaque Ca, Pi, F, and protein were measured.

There was a wide range of mineral formations seen by electron microscopy, mainly in the extracelluar matrix, and many were similar to those formed in natural plaque (Pearce *et al.*, 1991). We have counted 13 types so far. These include lines of mineral deposition which appear to relate to extensive intraplaque structures, possibly resulting from channels in the plaque, lipid structures, or extracellular polysaccharides. Possible associations with streptococcal walls were also seen. Clearly, intraplaque mineralization is a complex, multifaceted process. Microcosms may be the only useful *in vitro* systems available for the study of such unknown processes in detail.

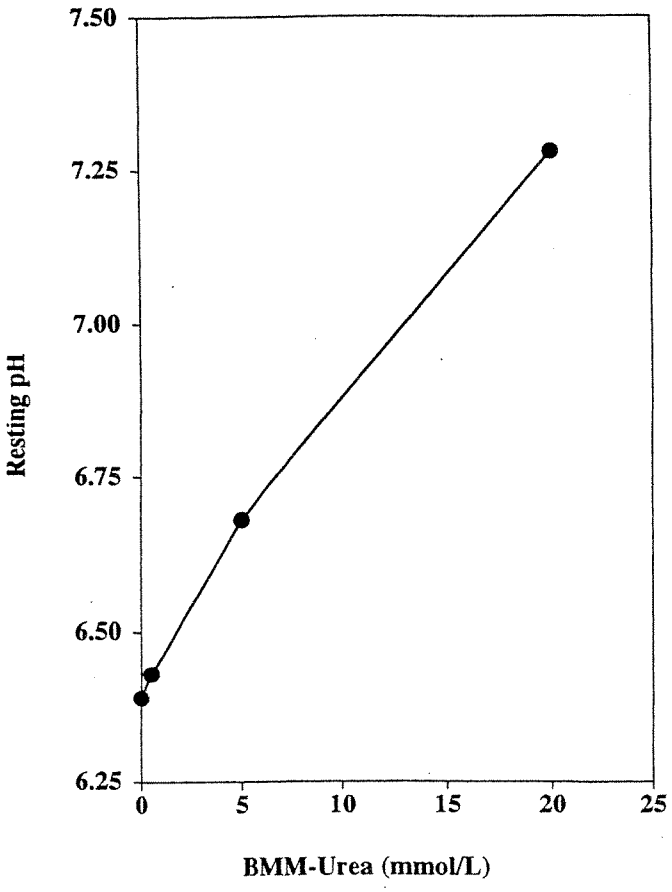

Fig. 3—Effect of supplementing BMM with urea at physiological concentrations on the resting pH.

A test system for plaque-modifying reagents. Plaque microcosms potentially give responses to interventions closely related to those of plaque *in vivo*. It is now well-established that bacterial resistance to insult is greater in plaque biofilms than in corresponding planktonic phases (ten Cate and Marsh, 1994; Bradshaw, 1995; Gilbert *et al.*, 1997; Marsh and Bradshaw, 1997). Mechanisms may include diffusion limitations, phenotypic adaptation, and selection of resistant species. We have examined the effect of ethanol on microcosm plaques at concentrations up to 40% (v/v), concentrations to which the oral cavity is regularly exposed in some people (Sissons *et al.*, 1996a). Examination of the minimum inhibitory concentration of ethanol to dispersed plaque bacteria enrichment cultures confirmed that no bacteria grew above 10% ethanol, while 40% ethanol sterilized them within 2 min. With 15-minute applications of ethanol every 4 hrs, the same microcosm biofilm was completely resistant to 10% ethanol, and much higher concentrations were needed for inhibition. If the ethanol inhibition was partially lifted by reducing the application time to 6 min on day 5 of growth, 30% ethanol treatments became ineffective, indicating some form of adaptation. Spontaneous resistance to antiseptics can arise within days (Sissons *et al.*, 1995a). In a biodiverse biofilm such as plaque, there are several mechanisms for development of resistance in response to environmental stress, allowing a homeostatic return to a functionally equivalent state. Evaluating these possibilities is important when in the development of procedures to reduce pathogenic species by antimicrobials.

Defined species consortia and microcosms are complementary model systems

The 'Marsh' nine-species consortia and microcosms

represent complementary synthetic and holistic approaches to plaque biofilm ecology and behavior (Table 10). They differ in their emphasis on complexity, biodiversity, relationship to plaque *in vivo* and plaque development, manipulability of the microflora, repeatability and reproducibility, and the prior knowledge needed of both the phenomenon being studied and its microbial basis.

Plaque reconstruction systems allow for a focus on processes which are basic to plaque biofilm function *per se* with near-total control and manipulation of the particular species present (Table 11A). Although the choice of strains and species is inevitably, to a degree, arbitrary, the effect of strain variation can be studied. The nine-species consortium has a biodiversity which is an order of magnitude greater than that of mono- and di-cultures, probably sufficient for higher levels of interaction and organization to emerge. This consortium provides a benchmark plaque community and a baseline for further development with potential to illuminate emergent properties of plaque biofilms and predict effects of antiplaque agents. Overall, this is an extremely powerful laboratory model of plaque which can be closely specified, modified, and analyzed, and which is applicable to the testing of hypotheses across almost the whole range of plaque biology.

The advantages of plaque microcosms are possibly more nebulous, and they have obvious disadvantages in their complex, heterogeneous, biodiverse, irrepeatable nature (Table 11B). Nevertheless, they are the laboratory model system most closely related to plaque *in vivo*. The biodiversity, heterogeneity, and complexity causing experimental difficulties are properties which characterize plaque *in vivo* and therefore need to be modeled in laboratory systems. Where consortia allow for control and manipulation of the environment for selected oral microflora, microcosms do so for an experimentally accessible natural plaque microflora. The concept of environmental control of evolution to convergent function in biodiverse systems reinforces the rationale for the use of microcosms and helps to explain plaque homeostasis and the flora changes in dental disease. Microcosms allow for the unexpected and unknown which is unsettling to predictions and experimental design. However, this unexpectedness, and high-level emergent properties, make microcosms a powerful system for the study of plaque behavior, ecology, and pathology and for the testing of potential clinical interventions. There are several studies where the attributes of microcosms may be especially advantageous (Table 12). There are now preliminary reports of several studies in addition to our research where plaque microcosms are being used (Palmer *et al.*, 1996a,b, 1997; Charbonneau *et al.* 1997; Wilson *et al.*, 1997). Overall, microcosm plaque biofilms make an unique and valuable contribution to the hierarchy of plaque model systems.

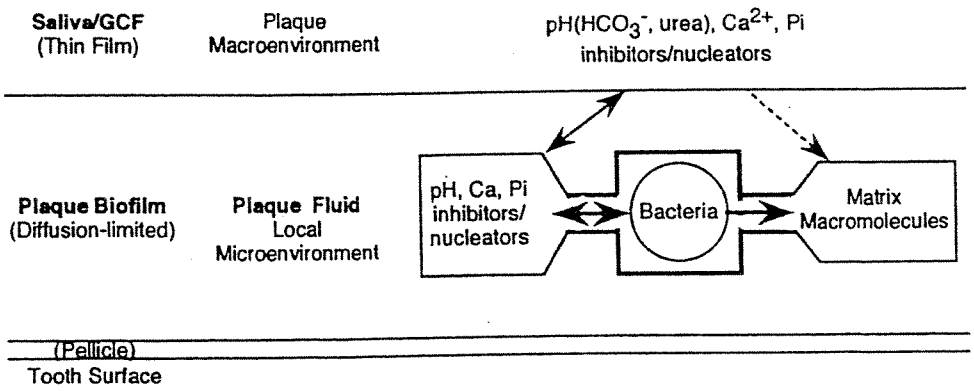

Fig. 4—Schematic diagram of some of the processes affecting intra-biofilm plaque mineralization.

CONCLUSION

There is a variety of plaque biofilm culture technologies suitable for different purposes and which complement

TABLE 10

FEATURES OF BIOFILM NINE-SPECIES CONSORTIUM AND MICROCOSM PLAQUE MODELS

Parameter	Consortium	Microcosm
Synthetic reconstruction	+	-
Holistic	-	+
Biodiversity	9	(100+?) x (clonotypes)
Control of species/clonotypes	total	almost none
Relationship to natural flora	limited/ arbitrary	is natural flora
Mature composition	*quasi* steady-state?	evolving
Reproducibility of		
Function—within expt	high?	CV* = 25%
—between expts	?	low?
Microflora composition	good?	low
Environmental control	high	high
Complexity	moderate	very high
Emergent/unexpected properties	modest	high

* CV is within-run Coefficient of Variation (Sissons *et al.*, 1995a,c).

TABLE 11

INVESTIGATIONS APPROPRIATE TO PLAQUE-LIKE CONSORTIA AND MICROCOSMS

(A) Plaque biofilm reconstruction consortia (n = 9):

* into basic biofilm physiology, mechanisms, processes and interactions (symbiosis, competition, antagonism); their environmental regulation and mode of action studies.
* requiring manipulation of:
  — plaque composition
  — plaque behavior
* where the required microbial species/functions are known to be represented in the consortium.

(B) Microcosms (*in vitro* evolved natural "plaque"):

* into plaque behavior, development, maturation, and reaction to environmental change and perturbation
* requiring:
  — the closest plaque-like *in vitro* system
  — allowance for unknown/unexpected processes
* where:
  — the exact microbial specificity is unknown
  — the processes involved are unknown
  — biodiversity may be important.

TABLE 12

POTENTIAL PLAQUE MICROCOSM STUDIES

Plaque growth, development, microbial succession.

Colonization resistance to invasion, commensal pathogens.

Holistic plaque behavior, *e.g.*, pH, calcification.

Response to: environmental factors, perturbation, insult.

Response to interventions/antimicrobial agents
— susceptibility, adaptation, selection for resistance.

Screening of antiplaque agents, anti-cariogenicity procedures.

Establishing quantitative parameters, *e.g.*, metabolic fluxes.

Confirmation of *in vivo* results in detail.

simpler and intra-oral appliance technologies. Realistic synthetic and holistic microcosm laboratory models are now being developed which enable studies to start addressing the complexity of plaque biofilm ecology and pathology, and realistically testing antiplaque effects of agents and procedures. Previously, such studies were confined to the difficult and uncontrollable environment of the mouth.

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